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Identification of Two Prokineticin cDNAs: Recombinant Proteins Potently Contract Gastrointestinal Smooth Muscle

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ABSTRACT

The motility of gastrointestinal tract is regulated by classical neurotransmitters, neuropeptides, and humoral agents. Two novel human cDNAs have been cloned based on their sequence similarity to a frog skin secretion protein, Bv8, and a nontoxic protein of mamba snake venom. These human cDNAs encode two secreted proteins of 86 and 81 amino acids. Northern blot hybridization has revealed that these cDNAs are expressed in gastrointestinal tract, particularly the stomach. Recombinant proteins with authentic N-terminal sequences have been produced in *Escherichia coli* and refolded into functional proteins by careful control of protein aggregation. Mass spectrometry has confirmed the formation of five pairs of disulfide bonds. The refolded recombinant proteins potently contract gastrointestinal smooth muscle with EC_{50} values in the subnanomolar range. The contractile effects of the recombinant proteins are specific for gastrointestinal smooth muscle, because they have no effect on vascular or respiratory smooth muscle. To reflect their potent and specific effects on gastrointestinal smooth muscle cells, we have named these recombinant proteins prokineticins. Ligand binding studies with iodinated prokineticin revealed the presence of a high-affinity site in ileal smooth muscle. The displacement of specific binding by GTPγS suggests that the prokineticin receptor may belong to the family of G protein-coupled receptors. Experiments with verapamil and nifedipine revealed that calcium influx is essential for the contractile activity of prokineticins on gastrointestinal smooth muscle. In summary, we have identified two novel endogenous regulators of gastrointestinal motility. The availability of recombinant prokineticins should provide novel therapeutic agents for disorders involving impaired gastrointestinal motility.

The function of gastrointestinal (GI) smooth muscle is to mix and propel intraluminal contents, enabling the efficient digestion of food, the progressive absorption of nutrients, and eventual evacuation of residual components. The activity of GI smooth muscle is regulated by intrinsic and extrinsic neural signals, including classical neurotransmitters, coexisting neuropeptides, and circulating peptide hormones (Fox-Threlked, 1993; Wood, 1994). Also, a number of locally produced humoral agents, including histamine, serotonin, and adenosine, influence the activity of smooth muscle cells (Burks, 1994). In addition to these endogenous agents, some exogenous peptides with contractile activity have been identified. Schweitz et al. (1990; 1999) purified a small protein from mamba venom [mamba intestinal toxin (MIT) 1] and showed that it potently stimulates contraction of the guinea pig ileum. Recently, a protein of similar size with greater than 40% identity with MIT1, including all 10 conserved cysteines, has been purified from frog skin secretion (Mollay et al., 1999). The frog protein, named Bv8, was also found to stimulate the contraction of GI smooth muscle with high potency (Mollay et al., 1999). Because a number of bioactive mammalian peptides or proteins, including bombesin, endothelin, natriuretic peptide, and the secreted form of PLA2, have found their counterparts in snake venom and frog skin secretion (McDonald et al., 1979; Brown et al., 1980; Takasaki et al., 1988; Yanagisawa et al., 1988; Schweitz et al., 1992; Tischfield, 1997), we sought to identify mammalian homolog(s) of frog Bv8 and snake MIT1 that may regulate the GI contractility.

Here, we describe the isolation and characterization of two human cDNAs that encode the homologs for snake MIT1 and frog Bv8. Refolded recombinant proteins were found to stimulate the contraction of gastrointestinal smooth muscle with high potency. We have named these proteins prokineticins to reflect their specific and potent contractile activity on GI smooth muscle. Evidence that prokineticins may interact with a G protein-coupled receptor family is also presented. The discovery of endogenous regulators of gastrointestinal motility should facilitate the development of novel therapeutics for disorders that involve impaired gastrointestinal motility.

ABBREVIATIONS: GI, gastrointestinal; MIT, mamba intestinal toxin; GST, glutathione-*S*-transferase; HPLC, high-performance liquid chromatography; RP, reverse(d) phase; EST, expressed sequence tag.

Materials and Methods

RNA Blot. Human multiple tissue RNA blots containing normalized samples of poly(A) RNA were used according to the manufacturer's instructions (CLONTECH, Palo Alto, CA). The blots were probed with random primer-labeled probes (nucleotides 1–550 and 1–1178 for prokineticin 1 and prokineticin 2 cDNAs), and signals were visualized by exposing to Kodak XAR film.

Cloning of Full-Length cDNAs. For cloning full-length prokineticin 2 cDNA, a 5′ RACE with human brain cDNA mixture (CLONTECH) was performed. The polymerase chain reaction conditions were 94°C for 30 s and 68°C for 2 min (30 cycles). The specific oligonucleotides used were RACE1: ACATGGGCAAGTGTGATGCAT and RACE2: ATTACTTTTGGGCTAAAC.

Production, Refolding, and Purification of Recombinant Prokineticins. The coding sequences for mature prokineticins were cloned into the prokaryotic expression vector pGEX-3X (Amersham Pharmacia Biotech, Piscataway, NJ). The extra nucleotides between the factor Xa protease digestion site of the glutathione-*S*-transferase (GST) tag and mature prokineticins were removed by site-directed mutagenesis and confirmed by sequencing. To facilitate protein purification, a 6xHis-tag was added to the C terminus so that the fusion proteins could be purified with Ni-NTA affinity chromatography (Qiagen, Valencia, CA). The detailed protocols for production of fusion proteins are as follows. *Escherichia coli* cells (BL21) were grown to absorbance 0.8 and induced with 600 μM isopropyl β-D-thiogalactoside for 2 h at 37°C. The cells were then pelleted, washed, and lysed with buffer A (6 M guanidine hydrochloride, 100 mM NaH_2PO_4, and 10 mM Tris, pH 8.0). Fusion proteins were allowed to bind to Ni-NTA beads and then washed extensively with buffer C (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 6.3) and buffer D (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 5.9). Fusion protein-bound beads were equilibrated with digestion buffer (50 mM Tris, 150 mM NaCl, and 1 mM $CaCl_2$, pH 7.5). Digestion was performed overnight at room temperature with 10 ng of protease factor Xa per microgram of fusion protein. The cleaved GST tag was then washed away with buffer D. Mature prokineticins were eluted with buffer E (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 4.5), and fractions were analyzed by SDS-PAGE. The pooled recombinant prokineticins were then refolded as follows. Proteins were diluted to 100 μg/ml with buffer E and dialyzed against renaturing buffer (4 M urea, 5 mM cysteine, 0.02% Tween-20, 10% glycerol, 10 mM Tris, 150 mM NaCl, 100 mM NaH_2PO_4, pH 8.3). New renaturing buffer (same as above except with 2 M urea) was then added, and dialysis was continued for 4 more days with at least one more change of renaturing buffer. The refolded protein was then desalted with a spin column (Qiagen) and analyzed by receptor binding or bioassay. The final purification was performed with reverse phase-HPLC (Amersham Pharmacia Biotech). Functional proteins were eluted with 0.08% trifluoroacetic acid and a 10 to 50% acetonitrile gradient. The elution of protein was monitored at 206 nm. Trifluoroacetic acid and acetonitrile were then evaporated by lyophilization.

Mass Spectrometry. The electrospray ionization mass spectrometry was performed with a 6.5-T HiResESI Fourier Transform mass spectrometer (IonSpec, Irvine, CA) as described previously (Li et al., 1994) with a sample volume of 100 μl. Protein eluted from RP-HPLC was lyophilized and dissolved in nanopure water and then diluted to a concentration of 1 μM with methanol/water/acetic acid (49.5%:49.5%:1%, v/v/v).

Isolated Smooth Muscle Preparations. Guinea pigs were euthanized with CO_2, and a section of ileum (2–3 cm) approximately 10 cm rostral to the cecum was removed. The tissue was washed clean with Krebs-Ringer-bicarbonate buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 10 mM glucose) and mounted longitudinally in an organ bath containing Krebs-Ringer-bicarbonate buffer. Isometric contractions were measured with a force-displacement transducer and polygraph as described previously (Thomas et al., 1993). The ileum was allowed to incubate for 1 h, and then three test doses of the muscarinic agonist oxotremorine-M were added to ensure that the contractions were reproducible and of sufficient magnitude. The ileum was washed and allowed to rest for 5 min between each test dose. The longitudinal fundic strip, zig-zag tracheal strip, and isolated colon (proximal and distal) were prepared as described previously (Thomas and Ehlert, 1996; Sawyer and Ehlert, 1998). Aortas and femoral arteries were dissected from adult rats and mounted in organ baths (10 ml) using procedures similar to those described above. Tension was recorded on a Grass polygraph with initial preloads of 0.5 g for intestinal and tracheal preparations and 2 g for aorta and femoral artery.

Iodination. Prokineticin 1 was iodinated by the iodogen method as described previously (Fraker and Speck, 1978). Briefly, refolded prokineticin 1 (7.5 μg) was incubated with 50 μg of iodogen in 50 μl of 0.5 M PBS, pH 7.2, for 15 min at room temperature. The reaction was stopped by transferring the mixture to a Microfuge tube containing 100 μl of PBS, 1 mM NaI, and 0.1% bovine serum albumin. Free iodine was removed by gel filtration on Bio-Gel P2, and the radioactivity in the void volume was measured. Assuming that all the radioactivity had been incorporated into 6.0 μg of prokineticin 1 (80% recovery rate), we calculate that the specific radioactivity as 372 Ci/mmol.

Receptor Binding. Membranes were prepared from guinea pig ileum as described (Li et al., 2000), except additional steps of differential centrifugation (800 g, 10,000 g, 100,000 g, 4°C, 20 min each) were applied to reduce the background binding. Incubation was performed in 4 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 0.1% bovine serum albumin at room temperature. For saturation binding, 1.5 to 200 pM labeled prokineticin 1 was used. Nonspecific binding was defined in the presence of 20 nM unlabeled prokineticin 1. For displacement experiments, unlabeled protein was preincubated with membrane in 3 ml of total reaction volume for 1 h, then ^{125}I-prokineticin 1 (20 pM) was added. The membrane was incubated for an additional 3 h at room temperature. The binding mixture was filtered through GF-C glass filters and washed with 10 ml of 20 mM Tris-HCl, pH 7.4. Radioactivity retained on filters was measured in a gamma counter. The data were analyzed with the LIGAND program.

Results

Identification and Analysis of Two Mammalian Homologs for Frog Bv8 and Snake MIT1. In an effort to identify mammalian homologs of frog Bv8 and snake MIT1, we searched multiple databases using the BLAST 2.1 algorithm (Altschul et al., 1997), with their protein sequences as queries. A search of the genome survey sequence and the high throughput genome sequence databases revealed a number of human bacterial artificial chromosome clones containing open reading frames homologous to Bv8 and MIT1. A further search of the EST database using the predicted human coding and 3′untranslated regions revealed the presence of two human EST sequences (ai277349 and aa883760). Sequence analysis revealed that aa883760 encodes a predicted protein (Heijne, 1986) with a signal peptide of 19 amino acids and a mature protein of 86 amino acids. Clone ai277349 was found to be a partial cDNA. Full-length sequence for EST clone ai277349, cloned by 5′ RACE with human brain cDNA as a template, was found to contain a signal peptide of 27 amino acids and a mature protein of 81 amino acids (Fig. 1). These proteins were named prokineticin 1 and prokineticin 2 (GenBank accession numbers AF333024 and AF333025), respectively (see below).

Sequence analysis reveals that prokineticins 1 and 2 con-

tain all 10 conserved cysteines and have about 44% amino acid identity. Both prokineticins possess about 43% identity with frog Bv8 and snake MIT1. Interestingly, the N-terminal sequences before the first cysteine (AVITGA) is completely conserved among all species (Fig. 1), suggesting a functional role for this region. Preliminary analysis of the mouse prokineticin 1 gene (data not shown) indicates that the N-terminal sequence AVITG is derived from the first exon that also contains the signal peptide sequence, whereas the cysteine-rich sequences are from other exon(s).

Prokineticins Are Expressed in GI and Other Tissues. As an initial survey of prokineticin expression, we probed a human master blot using fragments of human prokineticin cDNAs. Both prokineticins were widely expressed in various adult and fetal tissues, with a generally higher expression level of prokineticin 1 compared with prokineticin 2 (Fig. 2, A and B). The expression of prokineticins in peripheral tissues was further examined by Northern blot analysis. Figure 2D showed that prokineticin 1 is highly expressed in GI tissues, with the highest expression level in stomach, whereas prokineticin 2 expression can only be detected at low levels in small intestine. Interestingly, there are two different sizes of prokineticin 1 mRNA (1.5 and 1.8 kilobase pairs) among tissues examined, suggesting alternative splicing or alternative polyadenylation mechanisms for prokineticin 1.

Production, Refolding, and Purification of Human Prokineticins. Because the N-terminal sequences were completely conserved (Fig. 1), recombinant proteins with authentic N-terminal residue were produced first as GST-fusion protein, followed by the digestion with protease factor Xa to remove the GST tag. Figure 3 shows that a protein with correct molecular mass was produced by factor Xa digestion. Bioassay with the guinea pig ileum showed that the unfolded recombinant proteins were inactive (data not shown). As NMR examination indicated that the 10 cysteines of MIT1 are formed into five disulfide bonds (Boisbouvier et al., 1998) and that these 10 cysteines are all conserved in human prokineticin cDNAs, it seems likely that these disulfide bonds are probably essential for bioactivity. Thus, considerable effort was directed toward the attainment of the proper configuration of disulfide bonds (of 945 possible combinations). Initial refolding after a single dilution in refolding buffer was unsuccessful. Almost all of the recombinant proteins precipitated, probably because of the formation of intermolecular disulfide bonds. A series of modifications to control protein aggregation and to slow disulfide bond formation were then adopted (Georgiou and Valax, 1996; Lilie et al., 1998). These modifications included: 1) the reduction in the protein concentration to 100 μg/ml or less to favor the formation of intra- but not intermolecular disulfide bonds; 2) the use of dialysis instead of direct dilution; 3) the use of higher levels of urea (4 M and then 2 M) in all dialysis buffers; 4) the omission of the oxidants cystine or oxidized glutathione from redox pairs, leaving only 5 mM cysteine or 3 mM reduced glutathione; 5) the addition of glycerol to further reduce protein aggregation; and 6) the cooling of proteins and buffers to 4°C before initiating the refolding process. These carefully controlled steps allowed us to refold recombinant prokineticins successfully with minimal protein aggregation.

```
                                          ↓
A)              MRGATRVSIMLLLVTVSDC  AVITGA
B)  MRSLCCAPLLLLLLLPPLLLTPRAGDA  AVITGA
C)              MKCFAQIVVLLLVIAFSHG  AVITGA
D)                                   AVITGA

CERDVQCGAGTCCAISLWLRGLRMCTPLGREGEECHPG
CDKDSQCGGGMCCAVSIWVKSIRICTPMGKLGDSCHPL
CDKDVQCGSGTCCAASAWSRNIRFCIPLGNSGEDCHPA
CERDLQCGKGTCCAVSLWIKSVRVCTPVGTSGEDCHPA
*     *    **            *        *

SHKVPFFRKRKHHTCPCLPNLLCSRFPDGRYRCSMDLKNINF
TRKVPFFGRRMHHTCPCLPGLACLRTSFNRFICLAQK
SHKVPYDGKRLSSLCPCKSGLTCSK.SGEKFKCS
SHKIPFSGQRMHHTCPCAPNLACVQTSPKKFKCLSKS
                * *      *        *
```

Fig. 1. Amino acid sequences of prokineticin 1 (A), prokineticin 2 (B), Frog Bv8 (C), and partial sequence of MIT 1 (D). Ten conservative cysteine residues are marked (*). Signal peptides are underlined. Arrow indicates the splice sites for introns.

The refolded proteins were finally purified by RP-HPLC (Fig. 3, A, lane 5, and B). Mass spectrometry confirmed the formation of five disulfide bonds in refolded recombinant prokineticin 1. The molecular mass of 6xHis-tagged prokineticin 1, determined with a Fourier transform mass spectrometer, was found to be 10480.30 Da (Fig. 3C). Because the calculated molecular mass with all 10 cysteines present in reduced form was 10490.20, five pairs of disulfide bonds were clearly formed.

Refolded Recombinant Prokineticins Potently Contract Gastrointestinal Smooth Muscle. The refolded recombinant prokineticins were tested on isolated smooth muscle preparations. Figure 4 shows that both recombinant prokineticin 1 and prokineticin 2 potently stimulated the contraction of guinea pig ileum longitudinal muscle with EC_{50} values of about 0.46 and 0.90 nM, respectively. Prokineticin 1 (5 nM) also stimulated contraction of the fundic muscle strip and proximal colon, but had no effect on distal colon (25 nM, data not shown). Recombinant prokineticin 1 (25 nM) also had no effect on other smooth muscle tissues, including aorta, femoral artery, trachea, and gallbladder (data not shown). Thus, among the tissue tested, the contractile effect of prokineticins seems specific for GI smooth muscle.

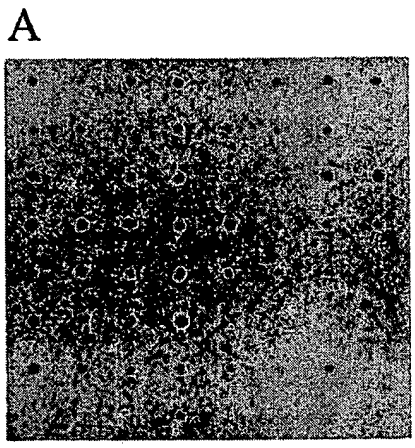
To investigate the possible signaling mechanisms of prokineticins, a number of kinase and ion channel inhibitors were tested. Tetrodotoxin, which is known to block nerve action potential propagation, had no effect on prokineticin 1-stimulated contractions of the longitudinal muscle of the ileum (Fig. 4B), indicating that prokineticin 1 acts directly on the smooth muscle. A number of compounds including the protein kinase C inhibitor calphostin C (1 μM), the phospholipase A2 inhibitor 7,7-dimethyl-(5*Z*,8*Z*)-eicosadienoic acid (10 μM), the tyrosine kinase inhibitor genistein (5 μM), the mitogen-activated protein kinase kinase inhibitor PD 098059 (10 μM), and the L-type calcium channel blockers verapamil and nifedipine were also investigated for their effects on prokineticin 1-induced contraction. Only the L-type calcium channel blockers were effective. At 100 nM, verapamil and nifedipine completely inhibited the contractile effect of 2 nM prokineticin 1 (Fig. 4C). The same concentration of verapamil and nifedipine also completely blocked the contractile action

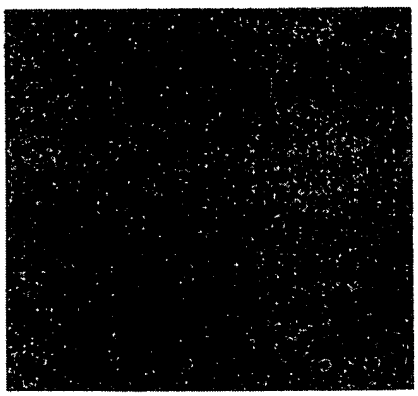
of the muscarinic agonist oxotremorine-M (100 nM) (Fig. 4F). This result indicates that, like muscarinic M3 receptor-mediated contraction of the ileum (Eglen et al., 1996; Ehlert et al., 1997), calcium entry via the voltage-gated calcium channel is an essential component of prokineticin signaling.

Bioactivities of Prokineticins Are Mediated by Membrane Receptors. The potent contractile action of recombinant prokineticins on guinea pig GI smooth muscle and the inhibitory effect of the calcium channel blockers suggest a receptor-mediated mechanism for prokineticins. To provide direct evidence that prokineticins are interacting with selective membrane receptors, we labeled recombinant prokineticin 1 with ^{125}I and carried out receptor binding experiments. Prokineticin 1 saturably labeled guinea pig ileum with high affinity. Scatchard analysis indicated that the specific binding of prokineticin 1 was best fitted with two-site model ($F = 38.78, P < 0.001$ verse one site model; Fig. 5A). The high- and low-affinity constants (K_d) were 5.0 ± 0.8 pM and 227 ± 63 pM ($n = 3$), respectively. The B_{max} for high- and low-affinity sites were 7.8 ± 1.2 and 26.4 ± 8.4 fmol/mg of protein, respectively ($n = 3$). Competition experiments revealed that the specific binding was displaced by recombinant prokineticin 1. The displacement curves were also best fitted with two-site model (with K_i of 8.0 ± 3.9 pM, and 1.50 ± 0.9 nM, $n = 3$ for high- and low-affinity sites, respectively) (Fig. 5B). Figure 5B also showed that prokineticin 2 displaced labeled prokineticin 1 with similar affinity (K_i of 4.2 pM for high affinity and 1.22 nM for low affinity site, average of two experiments). Because agonist binding to many G protein-coupled receptors is inhibited by GTP, we investigated whether GTPγS had any effect on specific ^{125}I-labeled prokineticin 1 binding. Figure 5B shows that GTPγS caused a concentration-dependent inhibition of ^{125}I-prokineticin 1 binding. At the highest concentration tested (10 μM), GTPγS displaced 85% of the specific prokineticin binding to ileal membranes. These results suggest that prokineticin receptor(s) may belong to the G protein-coupled receptor family.

Discussion

Our results unequivocally established the existence of mammalian homologs of frog Bv8 and snake MIT1. To reflect their potent and specific effects on GI smooth muscle, we have named these proteins prokineticins. Their high potency in specifically stimulating the contraction of guinea pig ileal smooth muscle but not other smooth muscles including aorta, femoral artery, trachea, and gallbladder indicate that prokineticins may be important endogenous regulators of GI motility. Prokineticins may regulate GI smooth muscle as neurocrine-signaling molecules, circulating hormones, or paracrine humoral agents (Fox-Threlked, 1993; Burks, 1994; Wood, 1994). Because prokineticins are also widely expressed outside the GI tract, it is possible that prokineticins may be released from remote organs and regulate GI activity. In this respect, the resistance of prokineticins to protease treatment (unpublished observations) may guarantee their potential





C

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cerebellum	cerebral cortex	frontal lobe	hippocampus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	nucleus accumbens	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA 100ng	yeast tRNA 100ng	E.coli rRNA 150ng	E.coli DNA 100ng	poly r(A) 100ng	human c-ot DNA 100ng	human DNA 100ng	human DNA 500ng

D

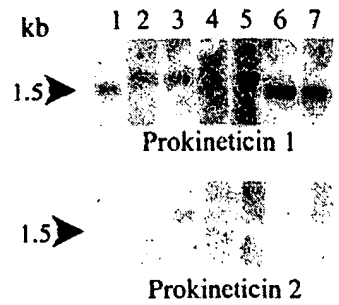


Fig. 2. Expression pattern of prokineticins. Human RNA master blot was probed with prokineticin 1 (A) and prokineticin 2 (B), respectively. C, diagram indicating the RNA sources for each dot. D, Northern blot analysis with prokineticin 1 and 2 in human peripheral tissues: 1, uterus; 2, colon; 3, small intestine; 4, bladder; 5, heart; 6, stomach; 7, prostate.

long-range and long-term effects. The molecular size and the processing of prokineticins distinguish them from typical neuropeptides, but render them more similar to cytokines (Loh et al., 1984; Vilcek, 1998). As one mechanism for eliminating pathogenic organisms is to enhance motility and push the offending organisms out of the GI tract, prokineticins may also be part of defending immune response (i.e., they function as inflammatory cytokines that increase the GI motility).

The high potency of recombinant prokineticins on the GI contractility suggests that prokineticins probably interact with cell surface receptor(s). This conclusion is reinforced by our receptor binding experiments, which demonstrate a saturable high affinity site for the iodinated recombinant prokineticin. Moreover, our observation that 10 μM GTPγS can displace almost all of the specific binding indicates the possible involvement of a G protein in prokineticin receptor signaling (Gilman, 1987; Gudermann et al., 1997). Moreover, the inhibitory effect of the calcium channel blockers verapamil and nifedipine on the contractile effect of prokineticin suggests a similar signaling mechanism for prokineticins and M3 muscarinic and motilin receptor in contracting GI smooth muscle: calcium entry via voltage-gated calcium channel is an essential component (Strunz et al., 1975; Eglen et al., 1996; Ehlert et al., 1997). Thus, the prokineticin receptor is likely to be a G protein-coupled receptor. However, other possibilities cannot be ruled out. For instance, prokineticins may cause smooth muscle contraction by directly activating

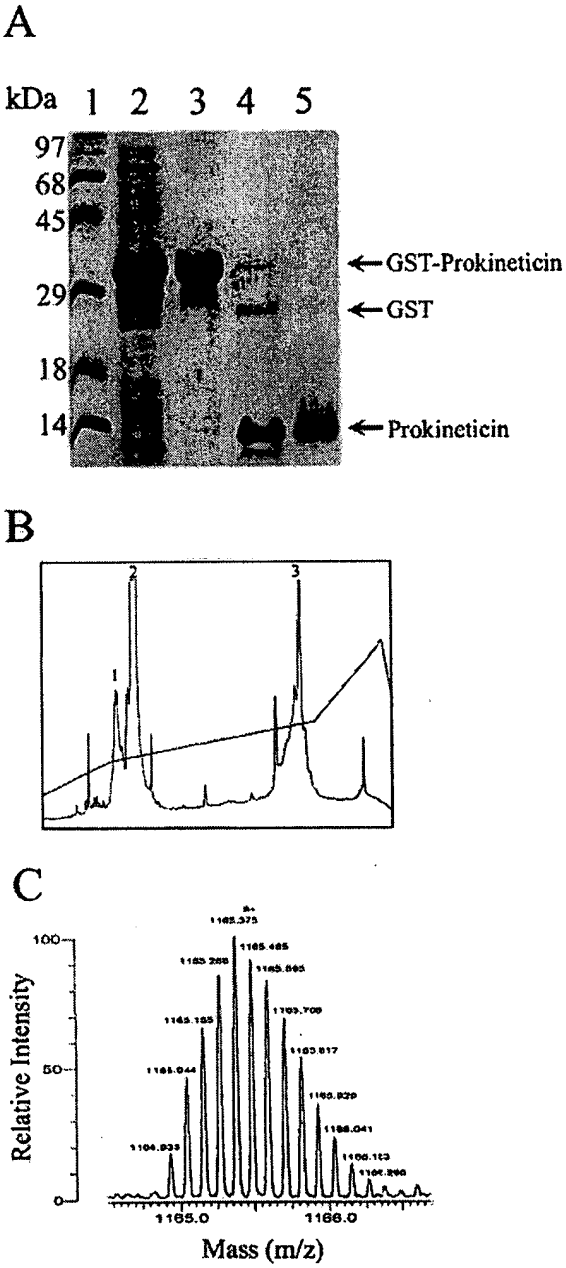


Fig. 3. Production and purification of human prokineticins. A, SDS-polyacrylamide gel electrophoresis (18%) of prokineticin 1 samples stained with Coomassie blue G-250. Lane 1, molecular mass standards; lane 2, whole cell lysate after induction; lane 3, Ni-NTA affinity chromatography-purified prokineticin; lane 4, factor Xa-digested prokineticin; lane 5, refolded prokineticin after HPLC purification. Each lane was loaded with 10 to 15 μg of total protein. B, reversed-phase HPLC separation of refolded protein mixture. Peak 2 contains refolded prokineticin. C, electrospray mass spectrum of refolded prokineticin 1 from peak 2 of RP-HPLC.

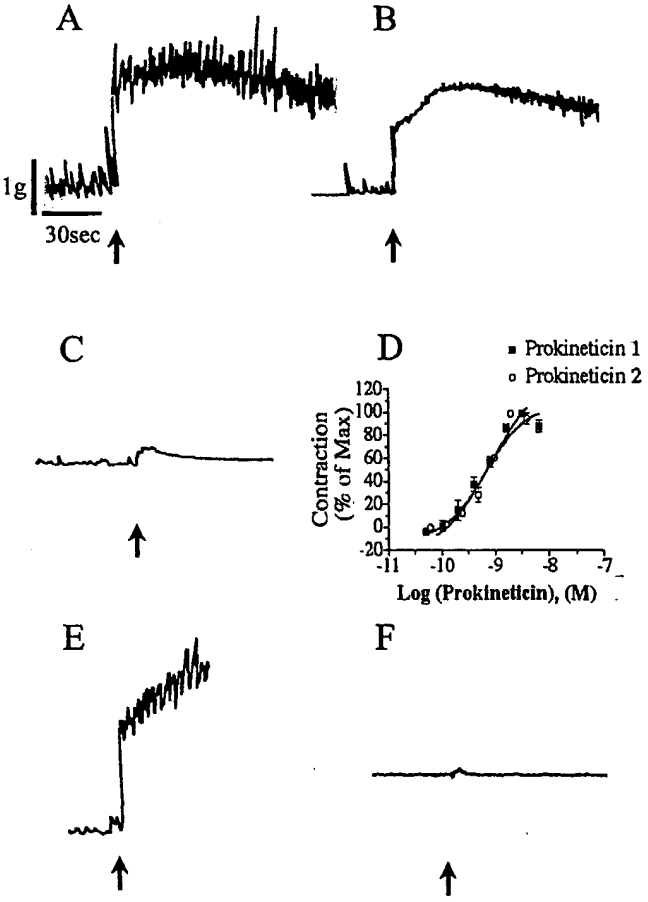


Fig. 4. Effects of prokineticins on the contractility of guinea pig ileal longitudinal muscle. The contractile responses to prokineticin 1 (2 nM) were measured in ileum in the absence (A) and in the presence of tetrodotoxin (0.1 μM; B) and verapamil (1 μM; C). D, concentration-response relationship for the contractile effects of prokineticins. Results are given as percentage of maximum contractility. Data are from three independent experiments. Contractile effects of oxotremorine-M in ileum in the absence (E) and in the presence of verapamil (1 μM; F) are also shown. Arrows indicate when drugs were added.

nonselective cation ion channels or by blocking inhibitory potassium channels on GI smooth muscle cells (Horowitz et al., 1996; Sanders, 1998). The availability of prokineticin receptor cDNA(s) should greatly facilitate the understanding of the prokineticin signaling mechanism.

Sequence analysis indicates that prokineticin may contain two functional domains—the short N terminus and the cysteine-rich C terminus. Because the N-terminal sequences preceding the first cysteine are completely conserved among prokineticins (Fig. 1), this region is likely to have functional importance. In addition to prokineticins and their isoforms from other species, a similar 10-cysteine motif is also found in a number of other secreted proteins including colipase, a cofactor for the intestinal lipid digestive enzyme lipase (van Tilbeurgh et al., 1992), and dickkopfs, a family of proteins that have an important role in early embryonic development (Aravind and Koonin, 1998; Glinka et al., 1998). Interestingly, dickkopfs actually possess two groups of 10-cysteine domains that have mirror symmetry. X-ray crystallography and solution structural analyses have clearly demonstrated that MIT1 has five pairs of disulfide bonds and is folded into a structure similar to colipase (Boisbouvier et al., 1998). Experiments with mutant and chimeric proteins should help to address the functional importance of prokineticin N-terminal and C-terminal domains. Wechselberger et al. (1999) have recently reported a mammalian cDNA sequence corresponding to prokineticin 2 here, but no functional studies were carried out. Interestingly, the cDNA sequence they reported has an insertion that encodes an extra 21 amino acids, suggesting the existence of alternative spliced form of prokineticin 2 in the testis. The functional significance of this alternative spliced form remains unclear.

To our knowledge, this is the first report of proteins with five pairs of disulfide bonds that are successfully refolded in vitro. Refolding of proteins with more than three pairs of disulfide bonds is still regarded as challenging and difficult (Georgiou and Valax, 1996; Lilie et al., 1998). The expression of such disulfide bond-rich proteins in *E. coli* often results in a lack of formation of disulfide bonds or, more probably, the formation of incorrect intramolecular or intermolecular disulfide bonds. These events routinely lead to the production of inactive recombinant proteins and their aggregation in bacterial inclusion bodies. In this study, we used a slow exchange method to refold prokineticins that contain five pairs of disulfide bonds. A number of factors eventually contributed to our successful refolding of prokineticins: 1) a slow rate of removal of denaturing agent; 2) the use of only reducing agents in the redox refolding mixture, thereby allowing the slow formation of disulfide bonds; 3) low temperature; 4) a high concentration of urea and glycerol in dialyzing buffer to prevent protein aggregation; 5) a low concentration of recombinant protein to favor the formation of intra- but not intermolecular disulfide bonds. These refolding conditions should be instrumental for the design of protocols for the refolding of other recombinant proteins possessing multiple disulfide bonds.

In summary, we have discovered two novel cDNAs encoding prokineticins. Refolded recombinant prokineticins potently and specifically stimulate the contraction of GI smooth muscle. Because impaired GI motility is a very common clinical manifestation in many disorders, including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, and gastroesophageal reflux disease (Tonini, 1996; Samsom and Smout, 1997; Achem and Robinson, 1998; Briejer et al., 1999), the discovery of an endogenous regulator of GI smooth muscle should facilitate the development of novel therapeutics for such disorders.

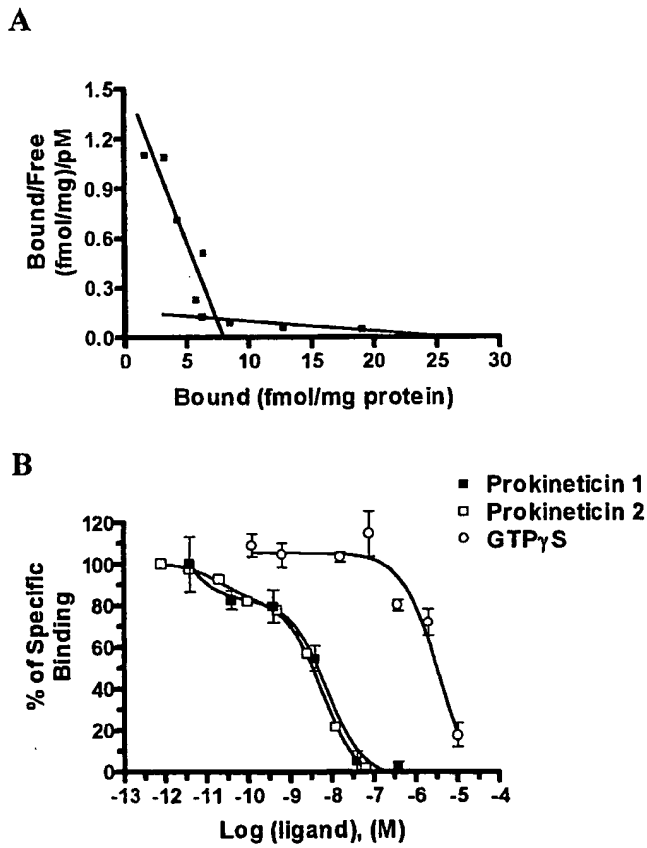


Fig. 5. A, Scatchard analysis of the specific binding of ^{125}I-prokineticin 1 to guinea pig ileal membranes. B, inhibitory effects of nonlabeled prokineticin 1 and GTPγS on the specific binding of ^{125}I-prokineticin 1 (20 pM) to a membrane preparation of the guinea pig ileum.

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